[redacted]

SUBJECT: Construct Hazard Analysis for TERA R-21-0001

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I. Introduction

The Agency has received a TSCA Environmental Release Application (TERA) from [redacted] to test [redacted] intergeneric strains of [redacted]. Table 1 below shows the case sub-numbers.

Table 1. Case sub-numbers for the [redacted] subject strains

[redacted]

The parental strain is described as [redacted]. This parental strain [redacted] was then transformed with a transient, [redacted]. This intermediate strain [redacted]) is considered the recipient strain for all [redacted] subject strains.

The [redacted] strains were engineered to [redacted]. All subject strains also contain the [redacted] gene, encoding [redacted] and used as [redacted].

[redacted] is seeking permission to conduct field testing of the [redacted] strains of [redacted] at [redacted] contract research organization (CRO) sites in [redacted] states. [redacted] intent is to perform experiments to determine the nitrogen-fixing abilities of the strains and how they affect plant uptake of nitrogen.

[redacted]

II. Genetic Construction of the Microorganism

Details of the genetic modifications done to arrive at the subject strains are presented in the Genetic Construction Report (Nguyen, 2021).

In summary, the parental [redacted] strain [redacted] was transformed with the [redacted], constitutively expressing an [redacted], [redacted] and the [redacted] gene from [redacted]. This intermediate, recipient strain [redacted] containing the [redacted]) was then transformed with one of the [redacted], which contains the [redacted] [redacted] the genes of interest. At this stage, the [redacted] was allowed to [redacted] the [redacted] by the [redacted], from the donor [redacted] to the [redacted] genome in a [redacted] step between [redacted]. Lastly, the resulting strains were [redacted] at a [redacted], allowing for [redacted] of both [redacted] and [redacted] plasmids, yielding the final subject strains.

This construction method resulted in the integration of the inter- and intra-generic genes listed in the table below.

Table 2. Genetic elements introduced into the subject strains (modified from TERA sec 2.2, Table 3)

[redacted]

**Although these genes are from the same genus as the recipient strain, DNA modifications were made to [redacted] for cloning purposes, therefore they are considered intergeneric.*

III. Potential Hazards Posed by the Genetic Modifications

Like the recipient strain, many in the genus [redacted] are known to be able [redacted] ([redacted]). Although listed as intergeneric due to modifications to [redacted], and [redacted] encode for the same functional proteins that already exist in the recipient [redacted] strain. The modifications in the [redacted] subject

[redacted]

microorganisms target an [redacted] and are not expected to change this involvement in context of all microorganisms in the soil that contribute to nitrogen fixation.

Table 2. Genetic elements introduced into the subject strains (modified from MCAN sec 2.2, Table 2)

[redacted]

A. *Inserted Genes*

Intergeneric genes

While the [redacted] gene is the only one that originated outside the [redacted] genus, other genes from [redacted]. used are also considered intergeneric due to DNA modifications [redacted] used during the [redacted] (Table 2). These include: [redacted] *and* [redacted] For [redacted]*),* there were no DNA modifications, therefor it is considered intrageneric.

[redacted]
In subject strains [redacted]**,** this operon codes for a [redacted], consisting of the [redacted], and [redacted] subunits [redacted] The [redacted] protein [redacted] molecules that are produced as part of central [redacted] thus [redacted]. The submitter hypothesized that overexpressing [redacted] [redacted].

For subject strain [redacted], this gene is driven by a constitutive, medium strength [redacted] promoter from [redacted]. For subject strain [redacted], this gene is driven by a constitutive, [redacted] from [redacted]. The submitter hypothesizes that the [redacted] promoter [redacted]. The [redacted] is used in both subject strains.

[redacted]

[redacted])
In the subject strain[redacted], this operon codes for an [redacted], consisting of [redacted] and [redacted] The [redacted] operon is involved in the formation of the [redacted] The submitter hypothesized that overexpressing the [redacted] operon may [redacted] thus increasing the capacity of the cells to fix nitrogen. The promoter used to overexpress this operon is the constitutive, medium strength [redacted] from [redacted]. The [redacted] from [redacted] is used for this operon.

[redacted])
In the subject strain [redacted], this operon, like [redacted] from [redacted], codes for an [redacted], but originates from [redacted] instead, consisting of [redacted], and [redacted]. The [redacted] operon is involved in the [redacted] The submitter hypothesized that overexpressing the [redacted] operon may increase availability of the [redacted] thus increasing the capacity of the cells to fix nitrogen. Although [redacted] operons (from [redacted] and [redacted]) exhibit high [redacted], the submitter anticipates that the [redacted] different operons may perform differently from each other when heterologously expressed in [redacted]. The promoter used to overexpress this operon is the constitutive, medium strength [redacted] from [redacted]. The [redacted] from [redacted] is used for this operon.

[redacted]
Present in subject strain[redacted], this codes for a [redacted] operon from [redacted], consisting of [redacted]). Individual genes are described below:

- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]

The submitter hypothesized that overexpression of the [redacted] operon may [redacted] such that it may be able to [redacted]

[redacted]

[redacted]. They also hypothesized that [redacted] will increase [redacted] activity above wild type levels when there are [redacted]. The promoter used to overexpress this operon is the [redacted] from [redacted]. The [redacted] from [redacted] is used for this operon.

[redacted]

In subject strain [redacted] this gene codes for a [redacted] from [redacted] [redacted]). [redacted] is involved in [redacted]. [redacted], which in turn [redacted] the [redacted] The submitter hypothesized that overexpression of [redacted] may [redacted] and thereby increase the capacity of the cells to reduce nitrogen. The promoter used to overexpress this gene is the constitutive, [redacted] from [redacted]. The [redacted] terminator from [redacted] is used for this gene.

[redacted]

In subject strain [redacted], this gene codes for a [redacted] from [redacted] [redacted]). Similar to [redacted] is involved in [redacted]. [redacted] also serves as the [redacted]. Under [redacted] conditions both [redacted] and [redacted] can perform this function but under [redacted] conditions only [redacted] can be active because [redacted] must be spatially or temporally separated from [redacted] The submitter hypothesized that overexpression of [redacted] may [redacted] and thereby increase the capacity of the cells to reduce nitrogen especially if the cells exist in [redacted]. The promoter used to overexpress this gene is the constitutive, [redacted], [redacted] from [redacted]. The [redacted] [redacted] from [redacted] is used for this gene.

Intrageneric genes

[redacted]

In subject strain [redacted], unlike the other [redacted] genes mentioned above, this [redacted] DNA sequence had not been modified prior to insertion, hence it is considered intrageneric. This operon codes [redacted], and [redacted]) proteins from [redacted]. These are homologous to the [redacted] and [redacted] proteins used to create subject strain [redacted]. Like [redacted] and [redacted] from [redacted], these serve the same functions, with [redacted] associated with [redacted] [redacted] and [redacted] encoding a [redacted]. The submitter hypothesizes that expressing these [redacted] and [redacted] proteins may aid in the [redacted] the [redacted], respectively. The promoter used to overexpress this gene is the constitutive, [redacted], [redacted] from [redacted]. The [redacted] from [redacted] is used for this gene.

[redacted]

Several [redacted] genes were elements of the [redacted] used to engineer the subject strains. However, the only one that remains in the final subject strains is the [redacted] gene that encodes [redacted] An [redacted] gene that encodes [redacted] and a [redacted] gene [redacted], encoding [redacted], were part of the [redacted] which does not remain in any of the final subject strains.

a. [redacted] gene (encodes [redacted])

This gene is located on all [redacted] as part of the [redacted] by the [redacted] and is integrated into each of the [redacted] subject strains. The [redacted] gene used in this TERA encodes a type [redacted] isolated from [redacted]; [redacted]). While [redacted] from [redacted], this gene is also found in a variety of bacteria ([redacted]). This [redacted] gene, including the promoter [redacted]) and terminator [redacted]) were [redacted] from a [redacted] (TERA Table 3) with primer [redacted] the [redacted] and [redacted]) as well as [redacted] cloning site (TERA Att. 2).

The [redacted] covalently modifies [redacted] by [redacted] the [redacted], thereby generating [redacted]). This resulting compound is ineffective as [redacted]. There are [redacted] of [redacted] proteins, [redacted], and [redacted] protein family members are present in many pathogens (e.g., *E. coli*, *Shigella*, *Salmonella enterica*) and have a high level of sequence similarity [redacted]

Per the TERA, this [redacted] gene was used for [redacted] of all the subject strains. The submitter also reports that all subject strains are [redacted] to [redacted] through the expression of the [redacted] gene.

Although [redacted] is considered by the World Health Organization as a highly important [redacted]) as is bactericidal for *Haemophilus influenza, Streptococcus pneumoniae,* and *Neisseria* meningitides, its potential toxicity has limited its use ([redacted] 4). Although rare, it can cause serious and fatal blood dyscrasias, including aplastic anemia, hypoplastic anemia, thrombocytopenia, and granulocytopenia [redacted]. It also can cause bone marrow suppression and a problem known as [redacted]. Because of the adverse side effects and the lack of ability to predict whether an individual will develop the serious blood disorders, it is only infrequently used in developed countries.

IV. Potential for Horizontal Gene Transfer

According to [redacted] is a genus comprised of [redacted], [redacted]. Members of the [redacted] genus are found in various environments, such as [redacted]), [redacted] [redacted]), insect larva ([redacted]), [redacted] [redacted]), and [redacted] ([redacted]). These bacteria have different biological characteristics that reflect a wide adaptation to

[redacted]

diverse environments. For example, [redacted] supports [redacted] ([redacted]).

Members of the genus [redacted]

With environmental introduction of genetically engineered microorganisms, the potential for horizontal gene transfer (HGT) of introduced genes into other microorganism in the environment warrants consideration. Horizontal gene transfer among bacteria is widespread and is responsible for acquisition of a myriad of traits in bacteria such as antibiotic resistance, xenobiotic degradation pathways, and even pathogenesis.

[redacted], have three well-documented mechanisms of horizontal gene transfer which are conjugation, transduction, and transformation (McClung, 2017). Although genomic evidence has shown [redacted]. to have undergone horizontal gene transfer events, [redacted] has mainly described as being on the recipient end ([redacted]). For example, through HGT, [redacted] is known to have great genetic diversity by acquiring foreign genes that allow its adaptation and survival [redacted]). This is also true for the closely related [redacted]., which are also known for their genomic diversity due to a state of natural competency, allowing for uptake of various survival genes [redacted]). Although naturally competent, the most likely transfer events include mobile genetic elements (MGEs) taken up by [redacted]. While [redacted] and [redacted]. are closely related, yet classified in different genera, horizontal gene transfer from [redacted] to [redacted]. is possible, but mainly through MGEs. Since the genes in this TERA are incorporated into the genome of [redacted] subject strains, there is low concern for transfer to [redacted].

Conjugation is the process whereby gene transfer occurs by means of plasmids or conjugative and integrated elements through close physical close contact between the donor and recipient cells. The DNA of the donor cell is transferred via a conjugation apparatus which consists of a conjugative pilus. In most bacteria, gene transfer by conjugation requires the formation of a mating pair formation complex and the DNA transfer and replication system (OECD, 2010).

Transduction is gene transfer mediated by bacteriophages whereby bacterial DNA can be transferred from a bacterial cell infected with a phage into a new host when the bacterial DNA is mistakenly packed into the empty phage head when the phage entity is produced. Then when the phage infects a new bacterial cell, the bacterial DNA in the phage from the donor is thus transferred into the recipient bacterial cell (OECD, 2010).

Transformation is the uptake of free DNA by "competent" bacterial cells. Competence is a genetically programmed physiological state of the recipient which requires elaborate machinery consisting of more than a dozen proteins (OECD, 2010). Natural competence has been shown to

[redacted]

be widespread among bacterial species (Dubnau, 1999) and more than 40 species of naturally transformable species were identified (Lorenz and Wakernagel, 1994).

The potential for HGT by conjugation and transduction must both be considered for any releases of viable cells from the fermentation facility as these two mechanisms require live cells. Of course, transformation must also be considered for releases of live cells that subsequently die and lyse, making their internal DNA available. In addition, the potential for horizontal gene transfer by transformation must be considered for the spent biomass if disposed of in the environment.

The inclusion of [redacted] genes as markers for selection of modified strains is not desirable for those microorganisms intended for, or having substantial potential for, environmental release. The potential hazard associated with the use of [redacted] genes is the [redacted] With intentional environmental release of microorganisms, as intended for the subject microorganisms in this TERA, there is much opportunity for horizontal gene transfer from introduced microorganisms to other microorganisms in the environment, particularly if those [redacted] genes reside on mobile genetic elements.

The [redacted], [redacted], present in subject microorganisms, are considered widely prevalent in natural microbial communities [redacted]), and the limited use of the subject microorganisms as proposed in this TERA is not expected significantly to increase the already existing pool of those genes in the environment. In addition, given that the markers are integrated into the bacterial chromosome and are stable, the risk of transfer of the [redacted] to other organisms is limited. Furthermore, [redacted] the [redacted] whose [redacted] was inserted in the submission microorganisms, are not of great [redacted].

Along with the [redacted] gene, all of the genetic material is stably integrated into the chromosome of the subject strains, which lessens the likelihood of horizontal gene transfer. The submitter also notes that no loss of the genetic material has been observed over numerous instances and many generations of culturing strains for research and development activities.

[redacted]

As mentioned in the Genetic Construction Report (Nguyen, 2021), the [redacted] method was used to incorporate the genes of interest into the recipient [redacted] strain to yield the fina[redacted] subject strains. This method has been well described and utilized for over 2 decades as a useful genetic engineering approach for [redacted]; [redacted] Per [redacted] the original [redacted] was a small [redacted] encoding a single [redacted], [redacted]. More recent studies have identified this element to be a member of a very diverse family of [redacted], all of which have been called [redacted]). They originate from a wide diversity of insects, as well as nematodes, flatworms and, recently, humans [redacted]). Very similar [redacted] have been found to occupy the genomes of species even in different phyla, indicating that these elements recently were horizontally transferred into their genomes [redacted]). [redacted]

[redacted]

[redacted].

Knowing these characteristics of these [redacted], this [redacted] method has become popular as a [redacted] approach, useful for engineering [redacted] microorganisms that may lack full genome sequencing/annotation [redacted]). Examples of this method's application have been shown for engineering *Clostridium ljungdahlii* for acetone production, and *Acidothiobacillus ferroxidans* for isobutyric acid biosynthesis ([redacted]). As with the [redacted] system utilized in this TERA, the [redacted] and incorporates [redacted] into the host genome [redacted]. This [redacted] does require additional effort to identify the [redacted] and also to ensure any disrupted genes do not interfere with the strain's overall fitness. [redacted] was able to identify these [redacted] genes through [redacted] as shown in section 2.2.4 of the TERA.

The genetic construction approach utilized a [redacted] where the [redacted] was on a [redacted] and the genes of interest flanked by the [redacted] were on another [redacted] (Nguyen, 2021). Once both were incorporated and [redacted] was allowed to take place, the [redacted] containing the [redacted] was no longer maintained in the subject strains. Since the [redacted] are specific to the [redacted], the genetic material [redacted] the [redacted] are not likely to be horizontally transferred to other microorganisms without the presence of the [redacted].

V. Conclusions

The recipient microorganism of genera [redacted] is not known to produce any toxins that might be harmful to humans, animals, or plants.

The genetic modifications made to the recipient microorganism introducing any of the genes used to create the subject strains, are not expected to impart or enhance any harmful traits beyond what may be present in the recipient strains. Although the [redacted] is present in all subject strains, this [redacted] is not currently [redacted]

The very small volume of the subject strains used in these field trials would not substantially add to that gene pool. The proposed field test of [redacted] strain [redacted], and [redacted] poses low concern for humans and the environment as the genetic modifications of introducing [redacted] and [redacted] also poses low hazards.

[redacted]

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